

FORM PTO-1390 (Modified) (REV 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER <b>RTSP-0262</b>	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR <b>10/069079</b>	
INTERNATIONAL APPLICATION NO. <b>PCT/US00/19747</b>		INTERNATIONAL FILING DATE <b>20 July 2000</b>		PRIORITY DATE CLAIMED <b>23 July 1999</b>	
TITLE OF INVENTION <b>ANTISENSE MODULATION OF MEKK1 EXPRESSION</b>					
APPLICANT(S) FOR DO/EO/US <b>MONIA, Brett P., GAARD, William A., WARD, Donna T., and COWSERT, Lex M.</b>					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.					
<ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.</li> <li>4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</li> <li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371 (c) (2)) <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</li> <li>b. <input type="checkbox"/> has been communicated by the International Bureau.</li> <li>c. <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> is attached hereto.</li> <li>b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</li> </ol> </li> <li>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)) <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</li> <li>b. <input type="checkbox"/> have been communicated by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).</li> <li>10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).</li> <li>11. <input checked="" type="checkbox"/> A copy of the International Preliminary Examination Report (PCT/IPEA/409).</li> <li>12. <input checked="" type="checkbox"/> A copy of the International Search Report (PCT/ISA/210).</li> </ol>					
<b>Items 13 to 20 below concern document(s) or information included:</b>					
<ol style="list-style-type: none"> <li>13. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li>14. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li>15. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.</li> <li>16. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</li> <li>17. <input type="checkbox"/> A substitute specification.</li> <li>18. <input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li>19. <input checked="" type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</li> <li>20. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</li> <li>21. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</li> <li>22. <input checked="" type="checkbox"/> Certificate of Mailing by Express Mail</li> <li>23. <input checked="" type="checkbox"/> Other items or information: <ol style="list-style-type: none"> <li>1) Courtesy copy of the International Application;</li> <li>2) Statement to support filing and submission in accordance with 37 CFR 1.821-1.825;</li> <li>3) Paper and disk copy of Amended Sequence Listing;</li> <li>4) Return post card.</li> </ol> </li> </ol>					



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.: RTSP-0262  
Inventors: Monia et al.  
Serial No.: Not yet assigned  
Filing Date: Herewith  
Examiner: Not yet assigned  
Group Art Unit: Not yet assigned  
Title: ANTISENSE MODULATION OF MEKK1 EXPRESSION

"Express Mail" Label No. EV 001201162 US  
Date of Deposit - January 18, 2002

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, DC 20231.

By Jane Massey Licata  
Typed Name: Jane Massey Licata, Reg. No. 32,257

BOX PCT

Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE  
WITH 37 CFR § 1.821 THROUGH 1.825

- ( ) I hereby state, in accordance with the requirements of 37 C.F.R. §1.821(f), that the contents of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 CFR §1.821(c) and (e), respectively are the same.
- ( ) I hereby state that the submission filed in accordance with 37 CFR §1.821(g) does not include new matter.
- ( ) I hereby state that the submission filed in accordance with 37 CFR §1.821(h) does not include new matter or go beyond the disclosure in the international application as filed.

- (XX) I hereby state that the amendments, made in accordance with **37 CFR §1.825(a)**, included in the substitute sheet(s) of the Sequence Listing were made to conform with the current Sequence Listing rules. I hereby state that the substitute sheet(s) of the Sequence Listing does not include new matter.
- (XX) I hereby state that the substitute copy of the computer readable form, submitted in accordance with **37 CFR §1.825(b)**, is the same as the amended Sequence Listing.
- ( ) I hereby state that the substitute copy of the computer readable form, submitted in accordance with **37 CFR §1.825(d)**, contains identical data to that originally filed.

Respectfully submitted,

*Jane Massey Licata*

Jane Massey Licata  
Registration No. 32,257

Date: January 18, 2002

Licata & Tyrrell P.C.  
66 E. Main Street  
Marlton, New Jersey 08053

(856) 810-1515

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Docket No.: RTSP-0262  
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Assistant Commissioner for Patents, Washington,  
D.C. 20231

By Jane Massey Licata  
Typed Name Jane Massey Licata, Reg. No. 32,257

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

PRELIMINARY AMENDMENT

It is respectfully requested that the Sequence Listing of the  
instant specification be deleted and replaced with the amended  
Sequence Listing provided herewith. A paper copy and a CRF copy of  
the amended Sequence Listing are provided herewith.

The replacement Sequence Listing has been amended to conform  
with the current Sequence Listing Rules.

Attorney Docket No.: RTSP-0262  
Inventors: Monia et al.  
Serial No.: Not yet assigned  
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No new matter has been added by this amendment.

Respectfully submitted,

*Jane Massey Licata*

Jane Massey Licata  
Registration No. 32,257

Date: January 18, 2002

Licata & Tyrrell P.C.  
66 E. Main Street  
Marlton, New Jersey 08053

(856) 810-1515

## SEQUENCE LISTING

<110> Brett P. Monia  
William Gaarde  
Donna T. Ward  
Lex M. Cowser  
ISIS PHARMACEUTICALS, INC.

<120> ANTISENSE MODULATION OF MEK1 EXPRESSION

<130> RTSP-0065

<150> 09/359,756

<151> 1999-07-23

<160> 47

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gaa aac caa atc att cac aga gat gtc aaa ggt gcc aat ttg cta att Glu Asn Gln Ile Ile His Arg Asp Val Lys Gly Ala Asn Leu Leu Ile 1345 1350 1355 1360	4080
gac agc act ggt cag aga cta aga att gca gat ttt gga gct gca gcc Asp Ser Thr Gly Gln Arg Leu Arg Ile Ala Asp Phe Gly Ala Ala Ala 1365 1370 1375	4128
agg ttg gca tca aaa gga act ggt gca gga gag ttt cag gga caa tta Arg Leu Ala Ser Lys Gly Thr Gly Ala Gly Glu Phe Gln Gly Gln Leu 1380 1385 1390	4176
ctg ggg aca att gca ttt atg gca cct gag gta cta aga ggt caa cag Leu Gly Thr Ile Ala Phe Met Ala Pro Glu Val Leu Arg Gly Gln Gln 1395 1400 1405	4224
tat gga agg agc tgt gat gta tgg agt gtt ggc tgt gct att ata gaa Tyr Gly Arg Ser Cys Asp Val Trp Ser Val Gly Cys Ala Ile Ile Glu 1410 1415 1420	4272

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atg gct tgt gca aaa cca cca tgg aat gca gaa aaa cac tcc aat cat 4320  
 Met Ala Cys Ala Lys Pro Pro Trp Asn Ala Glu Lys His Ser Asn His  
 1425 1430 1435 1440

ctt gct ttg ata ttt aag att gct agt gca act act gct cca tcg atc 4368  
 Leu Ala Leu Ile Phe Lys Ile Ala Ser Ala Thr Thr Ala Pro Ser Ile  
 1445 1450 1455

cct tca cat ttg tct cct ggt tta cga gat gtg gct ctt cgt tgt tta 4416  
 Pro Ser His Leu Ser Pro Gly Leu Arg Asp Val Ala Leu Arg Cys Leu  
 1460 1465 1470

gaa ctt caa cct cag gac aga cct cca tca aga gag cta ctg aag cat 4464  
 Glu Leu Gln Pro Gln Asp Arg Pro Pro Ser Arg Glu Leu Leu Lys His  
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cca gtc ttt cgt act aca tgg tag ccaattatac agatcaacta cgtagaaaca 4518  
 Pro Val Phe Arg Thr Thr Trp  
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gccactgaac agctatgaac gagggccagtg gggaaccctt acctaatgat gtgattgaca 4638

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20

&lt;210&gt; 7

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;223&gt; PCR Probe

&lt;400&gt; 7

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20

&lt;210&gt; 8

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 8

gctcctcgc cgccgctgc

20

&lt;210&gt; 9

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

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&lt;400&gt; 9

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20

&lt;210&gt; 10

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

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ctgtagtaac cattcttgca

20

<210> 22

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<213> Artificial Sequence



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&lt;400&gt; 22

tactgtagta accattcttg

20

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&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 23

ggtactgtag taaccattct

20

&lt;210&gt; 24

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 24

gggtactgt agtaaccatt

20

&lt;210&gt; 25

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 25

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&lt;212&gt; DNA

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&lt;210&gt; 27

&lt;211&gt; 20

&lt;212&gt; DNA

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20

&lt;210&gt; 28

&lt;211&gt; 20

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tagttgatct gtataattgg

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&lt;223&gt; Antisense Oligonucleotide

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&lt;210&gt; 46

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&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 46

ttttgcatac tgagcttagg

20

&lt;210&gt; 47

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 47

gcactagttt gggcttttgc

20

## ANTISENSE MODULATION OF MEKK1 EXPRESSION

## FIELD OF THE INVENTION

The present invention provides compositions and methods  
5 for modulating the expression of MEKK1. In particular,  
this invention relates to antisense compounds, particularly  
oligonucleotides, specifically hybridizable with nucleic  
acids encoding human MEKK1. Such oligonucleotides have  
been shown to modulate the expression of MEKK1.

## 10 BACKGROUND OF THE INVENTION

One of the principal mechanisms by which cellular  
regulation is effected is through the transduction of  
extracellular signals across the membrane that in turn  
modulate biochemical pathways within the cell. Protein  
15 phosphorylation represents one course by which  
intracellular signals are propagated from molecule to  
molecule resulting finally in a cellular response. These  
signal transduction cascades are highly regulated and often  
overlapping as evidenced by the existence of many protein  
20 kinases as well as protein phosphatases. It is currently  
believed that a number of disease states and/or disorders  
are a result of either aberrant expression or functional  
mutations in the molecular components of kinase cascades.  
Consequently, considerable attention has been devoted to  
25 the characterization of these proteins.

Nearly all cell surface receptors use one or more of the  
mitogen-activated protein kinase (MAP kinase) cascades  
during signal transduction. Three distinct subgroups of  
the MAP kinases have been identified and each of these  
30 consists of a specific module of downstream kinases. One  
subgroup of the MAP kinases is the Jun N-terminal  
kinase/Stress activated protein kinase (JNK/SAPK) cascade.  
This pathway was originally identified as an oncogene- and  
ultraviolet light-stimulated kinase pathway but is now  
35 known to be activated by growth factors, cytokines and T-

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cell costimulation (Moriguchi et al., *Adv. Pharmacol.*, 1996, 36, 121-137; Su and Karin, *Curr. Opin. Immunol.*, 1996, 8, 402-411).

MEKK1 (also known as mitogen-activated protein kinase  
5 kinase kinase 1, MEK kinase 1 and MAP/ERK kinase kinase 1)  
is a dual specific serine/threonine kinase that functions  
to mediate cellular responses to mitogenic stimuli. The  
MEKK1 protein has been shown to regulate signaling events  
associated with programmed cell death or apoptosis and NF-  
10 kappa-B, both of which have been associated with the  
development of hyperproliferative conditions such as  
cancer.

Apoptosis is not only a normal biological process but  
contributes to the pathogenesis of many diseases when  
15 deregulated. It has recently been shown that expression of  
the kinase domain of MEKK1 can potentiate the apoptotic  
response to external mitogenic stimuli in fibrosarcoma  
cells (Widmann et al., *Oncogene*, 1997, 15, 2439-2447).  
Further support of MEKK1 involvement in apoptosis comes  
20 from studies of Bcl-2, an intracellular protein that  
prevents apoptosis. These studies showed that Bcl-2  
prevented the mitogen-induced stimulation of MEKK1 in  
neuroglioma cells, indicating that MEKK1 lies directly  
downstream of Bcl-2 in an apoptosis signaling cascade (Park  
25 et al., *J. Biol. Chem.*, 1997, 272, 16725-16728).

Finally, studies involving the expression of mutant  
forms of MEKK1 protein corroborate the role for MEKK1 in  
apoptosis. Overexpression of a dominant negative form of  
MEKK1 was shown to prevent apoptosis induced by the  
30 topoisomerase inhibitor,  $\beta$ -lapachone (Shiah et al., *Cancer*  
*Res.*, 1999, 59, 391-398) and expression of a constitutively  
active mutant of MEKK1 has been shown to potentiate p53-  
dependent apoptosis (Fuchs et al., *Proc. Natl. Acad. Sci.*  
*U. S. A.*, 1998, 95, 10541-10546).

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MEKK1 has also been shown to play a critical role in the control of NF-kappa-B-mediated transcription at multiple points in the cascade (Meyer et al., *J. Biol. Chem.*, 1996, 271, 8971-8976). MEKK1 was shown to induce transcription  
5 from the human immunodeficiency virus-1 long terminal repeat and the interleukin-2R $\alpha$  promoters in a kappa-B dependent fashion (Meyer et al., *J. Biol. Chem.*, 1996, 271, 8971-8976). It was also shown to activate the I-kappa-B $\alpha$  kinase complex, an inhibitor of NF-kappa-B, through  
10 phosphorylation (Lee et al., *Cell*, 1997, 88, 213-222).

MEKK1 also participates in the signaling pathway of keratinocyte differentiation (Efimova et al., *J. Biol. Chem.*, 1998, 273, 24387-24395), tumor necrosis factor-alpha production in mast cells (Ishizuka et al., *Proc. Natl.*  
15 *Acad. Sci. U. S. A.*, 1997, 94, 6358-6363) and neurite outgrowth in PC12 cells (Leppa et al., *Embo. J.*, 1998, 17, 4404-4413) and has been shown to form complexes with JNK/SAPK and Grb2, through the MEKK1 noncatalytic N-terminus (Pomerance et al., *J. Biol. Chem.*, 1998, 273,  
20 24301-24304; Xu and Cobb, *J. Biol. Chem.*, 1997, 272, 32056-32060).

Currently, there are no known therapeutic agents which effectively inhibit the synthesis of MEKK1 and to date, strategies aimed at modulating MEKK1 function have involved  
25 the use of antibodies, dominant negative and dominant active mutants of the protein and antisense oligonucleotides. Disclosed in US Patent 5,405,941 are the protein encoded by the MEKK1 gene, antibodies to the protein (Johnson, 1995). United States Patent No. 5,981,265  
30 (Johnson) claims methods for regulating MEKK protein activity in a cell comprising transforming or transfecting the cell with a nucleic acid molecule encoding an MEKK protein such that MEKK protein activity in the cell is regulated, wherein the nucleic acid molecule is capable of



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hybridizing under stringent conditions to a nucleic acid molecule encoding MEKK1, MEKK2, MEKK3, MEKK4, MEKK5 or MEKK6. Oligonucleotides for use in antisense, and triplex formation, as ribozymes, probes or primers and in other applications are generally disclosed.

United States Patent No. 6,074,861 (Johnson) generally discloses isolated nucleic acid molecules capable of hybridizing under stringent conditions with an MEKK protein gene encoding an MEKK protein. Oligonucleotides for use in antisense, and triplex formation, as ribozymes, probes or primers and in other applications are generally disclosed.

Disclosed in PCT application WO 98/54203 are methods to increase cancer cell sensitivity to cancer therapy by contacting said cells with a MEKK1 inhibitor. These inhibitors being ribozymes, antisense nucleic acid molecules targeting MEKK1, or dominant negative mutants of MEKK1 (Mercola, 1998,). However, within this PCT publication, the composition of these inhibitors is not disclosed.

Therefore, there remains a long felt need for additional agents capable of effectively inhibiting MEKK1 function.

Antisense technology is emerging as an effective means for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of MEKK1 expression.

#### **SUMMARY OF THE INVENTION**

The present invention is directed to antisense compounds, particularly oligonucleotides, which are targeted to a nucleic acid encoding MEKK1, and which modulate the expression of MEKK1. Pharmaceutical and other compositions comprising the antisense compounds of the invention are also provided. Further provided are methods of modulating the expression of MEKK1 in cells or tissues comprising contacting said cells or tissues with one or

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more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression  
5 of MEKK1 by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric antisense  
10 compounds, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding MEKK1, ultimately modulating the amount of MEKK1 produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids  
15 encoding MEKK1. As used herein, the terms "target nucleic acid" and "nucleic acid encoding MEKK1" encompass DNA encoding MEKK1, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound  
20 with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include  
25 replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic  
30 activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of MEKK1. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition)  
35 in the expression of a gene. In the context of the present

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invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding MEKK1. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be

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preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the  
5 codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding MEKK1, regardless of the sequence(s) of such codons.

It is also known in the art that a translation  
10 termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of  
15 such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene  
20 that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the  
25 translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the  
30 translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3'  
35 direction from the translation termination codon, and thus

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including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between

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two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA  
5 are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus,  
10 "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the  
15 sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal  
20 function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under  
25 physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense  
30 oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a  
35 biological pathway. Antisense modulation has, therefore,

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been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as  
5 therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic  
10 modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of  
15 ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions  
20 which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of  
25 nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below.  
30 The antisense compounds in accordance with this invention preferably comprise from about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising  
35 from about 12 to about 25 nucleobases. As is known in the

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art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are

5 nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming

10 oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are

15 generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

20 Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones

25 include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside

30 backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates

35 including 3'-alkylene phosphonates and chiral phosphonates,



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phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having  
5 normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

10 Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131;  
15 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

20 Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain  
25 heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene  
30 formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts.

35 Representative United States patents that teach the

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preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

10 In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such  
15 oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular  
20 an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited  
25 to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, 1991, 254, 1497-1500.

Most preferred embodiments of the invention are  
30 oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular  $-CH_2-NH-O-CH_2-$ ,  $-CH_2-N(CH_3)-O-CH_2-$  [known as a methylene (methylimino) or MMI backbone],  $-CH_2-O-N(CH_3)-CH_2-$ ,  $-CH_2-N(CH_3)-N(CH_3)-CH_2-$  and  $-O-N(CH_3)-CH_2-CH_2-$  [wherein the  
35 native phosphodiester backbone is represented as

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-O-P-O-CH<sub>2</sub>-] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C<sub>1</sub> to C<sub>10</sub> alkyl or C<sub>2</sub> to C<sub>10</sub> alkenyl and alkynyl. Particularly preferred are O[(CH<sub>2</sub>)<sub>n</sub>O]<sub>m</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>OCH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>NH<sub>2</sub>, O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>ONH<sub>2</sub>, and O(CH<sub>2</sub>)<sub>n</sub>ON[(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>]<sub>2</sub>, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C<sub>1</sub> to C<sub>10</sub> lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxy, i.e., a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>3</sub>)<sub>2</sub>, also described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O-

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-

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azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by

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reference.

- Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which
- 5 enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med.*
- 10 *Chem. Lett.*, 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20, 533-538), an aliphatic
- 15 chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327-330; Svinarchuk et al., *Biochimie*, 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-
- 20 hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969-973), or adamantane acetic acid
- 25 (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxysterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923-937).
- 30 Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802;

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5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718;  
5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779;  
4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582;  
4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830;  
5 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506;  
5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241,  
5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667;  
5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481;  
5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and  
10 5,688,941, certain of which are commonly owned with the  
instant application, and each of which is herein  
incorporated by reference.

It is not necessary for all positions in a given  
compound to be uniformly modified, and in fact more than  
15 one of the aforementioned modifications may be incorporated  
in a single compound or even at a single nucleoside within  
an oligonucleotide. The present invention also includes  
antisense compounds which are chimeric compounds.

"Chimeric" antisense compounds or "chimeras," in the  
20 context of this invention, are antisense compounds,  
particularly oligonucleotides, which contain two or more  
chemically distinct regions, each made up of at least one  
monomer unit, i.e., a nucleotide in the case of an  
oligonucleotide compound. These oligonucleotides typically  
25 contain at least one region wherein the oligonucleotide is  
modified so as to confer upon the oligonucleotide increased  
resistance to nuclease degradation, increased cellular  
uptake, and/or increased binding affinity for the target  
nucleic acid. An additional region of the oligonucleotide  
30 may serve as a substrate for enzymes capable of cleaving  
RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is  
a cellular endonuclease which cleaves the RNA strand of an  
RNA:DNA duplex. Activation of RNase H, therefore, results  
in cleavage of the RNA target, thereby greatly enhancing  
35 the efficiency of oligonucleotide inhibition of gene

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expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target  
5 region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be  
10 formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United  
15 States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.:  
5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878;  
5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355;  
5,652,356; and 5,700,922, certain of which are commonly  
20 owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis.  
25 Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides  
30 such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules.  
35 The compounds of the invention may also be admixed,



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encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.:  
5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291;  
10 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330;  
4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170;  
5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978;  
5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259;  
5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of  
15 which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing  
20 (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and  
25 other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or  
30 conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphatate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 to Imbach et  
35 al.

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The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric

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- acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid,
- 5 methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid,
- 10 nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid,
- 15 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfoic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of
- 20 cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and
- 25 include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

- For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not
- 30 limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric
- 35 acid and the like; (c) salts formed with organic acids

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such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, 5 p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

10 The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression 15 of MEKK1 is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. 20 Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for 25 research and diagnostics, because these compounds hybridize to nucleic acids encoding MEKK1, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding MEKK1 can be 30 detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of MEKK1 in a sample may also be 35 prepared.

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The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered  
5 in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or  
10 insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or  
15 infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical  
20 administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the  
25 like may also be useful.

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers,  
30 dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not  
35 limited to, penetration enhancers, carrier compounds and

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other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may  
5 be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit  
10 dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared  
15 by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be  
20 formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions  
25 may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the  
30 pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the  
35 consistency of the final product. The preparation of such

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compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

## 5 Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1  $\mu\text{m}$  in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and

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anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and  
5 water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a  
10 system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or  
15 discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style  
20 ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers,  
25 absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active  
30 agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*,  
35 Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc.,



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New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage*

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Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as

5 polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for  
10 example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

15 Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations  
20 include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical  
25 scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

30 The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York,  
35 N.Y., volume 1, p. 199). Emulsion formulations for oral

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delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is

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dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack

5 Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical*  
10 *Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional  
15 emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions  
20 include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500),  
25 decaglycerol monocaprates (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves  
30 to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-  
35 emulsifying microemulsion systems are known in the art.

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The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not  
5 limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils  
10 and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral  
15 bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from  
20 enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity  
25 (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J. Pharm. Sci.*, 1996, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile  
30 drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will

5 within the gastrointestinal tract, vagina, buccal cavity  
and other areas of administration.

10 to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, 15 fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed above.

## Liposomes

20        There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and  
25        the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages

in vivo.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into

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the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., *Biochem. Biophys. Res. Commun.*, 1987, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed



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from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, 1992, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., *Antiviral Research*, 1992, 18, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. *S.T.P. Pharma. Sci.*, 1994, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming

lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside  $G_{M1}$ , or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen et al., *FEBS Letters*, 1987, 223, 42; Wu et al., *Cancer Research*, 1993, 53, 3765). Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al. (*Ann. N.Y. Acad. Sci.*, 1987, 507, 64) reported the ability of monosialoganglioside  $G_{M1}$ , galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al. (*Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside  $G_{M1}$  or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (*Bull. Chem. Soc. Jpn.*, 1980, 53, 2778) described liposomes comprising a nonionic detergent,  $2C_{12}15G$ , that contains a PEG moiety. Illum et al. (*FEBS Lett.*, 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood

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half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov et al. (*FEBS Lett.*, 5 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (*Biochimica et Biophysica Acta*, 1990, 1029, 91) extended 10 such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 15 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al. (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Patent No. 5,213,804 and European 20 Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin et al.) and in WO 94/20073 (Zalipsky et al.) Liposomes comprising PEG-modified ceramide lipids are described in WO 25 96/10391 (Choi et al.). U.S. Patent Nos. 5,540,935 (Miyazaki et al.) and 5,556,948 (Tagawa et al.) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids 30 are known in the art. WO 96/40062 to Thierry et al. discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa et al. discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an

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antisense RNA. U.S. Patent No. 5,665,710 to Rahman et al. describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love et al. discloses liposomes comprising antisense  
5 oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly  
10 deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g. they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their  
15 targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of  
20 serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The  
25 most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for  
30 categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants  
35 find wide application in pharmaceutical and cosmetic

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products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol  
5 esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block  
10 polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include  
15 carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important  
20 members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include  
25 quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is  
30 classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in  
35 *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York,

NY, 1988, p. 285).

#### Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention; surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi et al., *J. Pharm.*

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Pharmacol., 1988, 40, 252).

- Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid),
- 5 myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines,
- 10 C<sub>1-10</sub> alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92; Muranishi,
- 15 *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; El Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44, 651-654).

- Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and
- 20 fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term
- 25 "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium
- 30 dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate),

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- chenodeoxycholic acid (sodium chenodeoxycholate),  
ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-  
fusidate (STDHF), sodium glycodihydrofusidate and  
polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical*  
5 *Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92;  
Swinyard, Chapter 39 In: *Remington's Pharmaceutical*  
*Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co.,  
Easton, PA, 1990, pages 782-783; Muranishi, *Critical*  
*Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33;  
10 Yamamoto et al., *J. Pharm. Exp. Ther.*, 1992, 263, 25;  
Yamashita et al., *J. Pharm. Sci.*, 1990, 79, 579-583).

- Chelating Agents: Chelating agents, as used in  
connection with the present invention, can be defined as  
compounds that remove metallic ions from solution by  
15 forming complexes therewith, with the result that  
absorption of oligonucleotides through the mucosa is  
enhanced. With regards to their use as penetration  
enhancers in the present invention, chelating agents have  
the added advantage of also serving as DNase inhibitors, as  
20 most characterized DNA nucleases require a divalent metal  
ion for catalysis and are thus inhibited by chelating  
agents (Jarrett, *J. Chromatogr.*, 1993, 618, 315-339).  
Chelating agents of the invention include but are not  
limited to disodium ethylenediaminetetraacetate (EDTA),  
25 citric acid, salicylates (e.g., sodium salicylate, 5-  
methoxysalicylate and homovanilate), N-acyl derivatives of  
collagen, laureth-9 and N-amino acyl derivatives of beta-  
diketones (enamines) (Lee et al., *Critical Reviews in*  
*Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi,  
30 *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990,  
7, 1-33; Buur et al., *J. Control Rel.*, 1990, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-  
chelating non-surfactant penetration enhancing compounds  
can be defined as compounds that demonstrate insignificant



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activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33). This

5 class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and non-

10 steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, 1987, 39, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example,

15 cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

20 Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

25 Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity *per se*) but is recognized

30 as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier

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compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition  
5 between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-  
10 4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res. Dev.*, 1995, 5, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 1996, 6, 177-183).

#### Excipients

15 In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is  
20 selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to,  
25 binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen  
30 phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch

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glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which  
5 do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose,  
10 magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or  
15 solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with  
20 nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin,  
25 hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at  
30 their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional  
35 materials useful in physically formulating various dosage

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forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 1206-1228). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in

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compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents  
5 are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic  
10 acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their  
15 subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution  
20 of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on  
25 the relative potency of individual oligonucleotides, and can generally be estimated based on  $EC_{50}$ s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly,  
30 monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be  
35 desirable to have the patient undergo maintenance therapy

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to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

5 While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

## 10 EXAMPLES

### Example 1

#### Nucleoside Phosphoramidites for Oligonucleotide Synthesis Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl  
15 phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides  
20 synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine  
25 (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, 1993, 21, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

### 30 2'-Fluoro amidites

#### 2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, 1993, 36, 831-841] and United States patent 5,670,633,

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herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by  
5 modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S<sub>N</sub>2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of  
10 the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

#### 2'-Fluorodeoxyguanosine

15 The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group  
20 was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups.  
25 Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

#### 2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure  
30 in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'-phosphoramidites.

#### 2'-Fluorodeoxycytidine

35 2'-deoxy-2'-fluorocytidine was synthesized via

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amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

5        **2'-O-(2-Methoxyethyl) modified amidites**

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

10        **2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]**

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture  
15 was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The  
20 product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to  
25 give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a  
30 gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

**2'-O-Methoxyethyl-5-methyluridine**

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol



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(1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH<sub>3</sub>CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH<sub>2</sub>Cl<sub>2</sub>/acetone/MeOH (20:5:3) containing 0.5% Et<sub>3</sub>NH. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

**2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH<sub>3</sub>CN (200 mL). The residue was dissolved in CHCl<sub>3</sub> (1.5 L) and extracted with 2x500 mL of saturated NaHCO<sub>3</sub> and 2x500 mL of saturated NaCl. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated. 275 g of residue was obtained.

The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et<sub>3</sub>NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

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**3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture  
5 prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as  
10 judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl<sub>3</sub> (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl<sub>3</sub>. The  
15 combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was  
20 recovered from later fractions.

**3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine**

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH<sub>3</sub>CN (700 mL) and set  
25 aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH<sub>3</sub>CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl<sub>3</sub> was added dropwise, over a 30 minute period, to the stirred  
30 solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the  
35 reaction mixture and the solution was evaporated. The

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residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO<sub>3</sub> and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was  
5 triturated with EtOAc to give the title compound.

**2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M)  
10 in dioxane (500 mL) and NH<sub>4</sub>OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400  
15 mL) saturated with NH<sub>3</sub> gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over  
20 sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

**N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-  
25 cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL).  
30 The residue was dissolved in CHCl<sub>3</sub> (700 mL) and extracted with saturated NaHCO<sub>3</sub> (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO<sub>4</sub> and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et<sub>3</sub>NH as the  
35 eluting solvent. The pure product fractions were

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evaporated to give 90 g (90%) of the title compound.

**N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite**

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO<sub>3</sub> (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH<sub>2</sub>Cl<sub>2</sub> (300 mL), and the extracts were combined, dried over MgSO<sub>4</sub>, and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

**2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites**

**2'-(Dimethylaminooxyethoxy) nucleoside amidites**

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

**5'-O-tert-Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine**

O<sup>2</sup>-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500

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ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient  
5 temperature. TLC (*R<sub>f</sub>* 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium  
10 bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline  
15 product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-*tert*-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

20 In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-*tert*-  
25 Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100  
30 psig). The reaction vessel was cooled to ambient and opened. TLC (*R<sub>f</sub>* 0.67 for desired product and *R<sub>f</sub>* 0.82 for *ara*-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped,  
35 concentrated under reduced pressure (10 to 1mm Hg) in a

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warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butylldiphenylsilyl-5-methyluridine

5'-O-tert-Butylldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P<sub>2</sub>O<sub>5</sub> under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-butylldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

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5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-[(2-phthalimidooxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry  $\text{CH}_2\text{Cl}_2$  (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at  $-10^\circ\text{C}$  to  $0^\circ\text{C}$ . After 1 h the mixture was filtered, the filtrate was washed with ice cold  $\text{CH}_2\text{Cl}_2$  and the combined organic phase was washed with water, brine and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-tert-butyl-  
10 diphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-tert-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

5'-O-tert-butyl-  
20 diphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at  $10^\circ\text{C}$  under inert atmosphere. The reaction  
25 mixture was stirred for 10 minutes at  $10^\circ\text{C}$ . After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in  $\text{CH}_2\text{Cl}_2$ ). Aqueous  $\text{NaHCO}_3$  solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl  
30 acetate phase was dried over anhydrous  $\text{Na}_2\text{SO}_4$ , evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to  $10^\circ\text{C}$

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in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO<sub>3</sub> (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to get 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

**2'-O-(dimethylaminoxyethyl)-5-methyluridine**

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-tert-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to get 2'-O-(dimethylaminoxyethyl)-5-methyluridine (766mg, 92.5%).

**5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine**

2'-O-(dimethylaminoxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> (containing a few drops of pyridine) to get 5'-O-DMT-



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2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5 5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C. Then the reaction mixture was dissolved  
10 in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate  
15 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous NaHCO<sub>3</sub> (40mL). Ethyl acetate layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-  
20 2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in  
25 the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-  
30 ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside

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may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinasso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

**2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites**

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>2</sub>)<sub>2</sub>, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

**2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine**

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O<sup>2</sup>-, 2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude

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solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (2x200 mL). The combined CH<sub>2</sub>Cl<sub>2</sub> layers are washed with saturated NaHCO<sub>3</sub> solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH<sub>2</sub>Cl<sub>2</sub>:Et<sub>3</sub>N (20:1, v/v, with 1% triethylamine) gives the title compound.

25 5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by

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silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

**Example 2****Oligonucleotide synthesis**

5        Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

10        Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the  
15        capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution.        Phosphinate oligonucleotides are prepared as  
20        described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

25        3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent  
30        5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

35        3'-Deoxy-3'-amino phosphoramidate oligonucleotides are

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prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by  
5 reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

#### Example 3

#### 10 Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino  
15 linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are  
20 prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and  
25 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

#### Example 4

#### 30 PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*,

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1996, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

#### Example 5

#### 5 Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is  
10 positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped  
15 oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

#### [2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric

#### Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl  
20 phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphor-  
25 amidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The  
30 fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect

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all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,4-dithiolane-2-one 1,1 dioxides (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

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**Example 6****Oligonucleotide Isolation**

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by <sup>31</sup>P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, 266, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

**Example 7****Oligonucleotide Synthesis - 96 Well Plate Format**

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,4-dihydro-2H-benzodithiole-3-one 1,1-dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base



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protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated  $\text{NH}_4\text{OH}$  at elevated temperature (55-60°C) for 12-16 hours and the released product then  
5 dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

#### Example 8

##### 10 Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in  
15 either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test  
20 plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

#### Example 9

##### 25 Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using,  
30 for example, PCR or Northern blot analysis. The following four cell types are provided for illustrative purposes, but other cell types can be routinely used.

##### T-24 cells:

The transitional cell bladder carcinoma cell line T-24  
35 was obtained from the American Type Culture Collection

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(ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD),  
5 penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a  
10 density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

15 A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD)  
20 supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached  
25 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium  
30 (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from  
35 the Clonetics Corporation (Walkersville MD). HEKs were

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5 routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200  $\mu$ L OPTI-MEM™-1 reduced-  
10 serum medium (Gibco BRL) and then treated with 130  $\mu$ L of OPTI-MEM™-1 containing 3.75  $\mu$ g/mL LIPOFECTIN™ (Gibco BRL) and the desired oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16 hours  
15 after oligonucleotide treatment.

#### Example 10

#### Analysis of oligonucleotide inhibition of MEKK1 expression

Antisense modulation of MEKK1 expression can be assayed in a variety of ways known in the art. For  
20 example, MEKK1 mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA.  
25 Methods of RNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern blot analysis is routine in the art and is taught in, for example, Ausubel,  
30 F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems,

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Foster City, CA and used according to manufacturer's instructions. Other methods of PCR are also known in the art.

MEKK1 protein levels can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to MEKK1 can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

#### Example 11

##### Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, 1996, 42, 1758-1764. Other methods for

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poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200  $\mu$ L cold PBS. 60  $\mu$ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55  $\mu$ L of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200  $\mu$ L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60  $\mu$ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

#### Example 12

#### 25 Total RNA Isolation

Total mRNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200  $\mu$ L cold PBS. 100  $\mu$ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100  $\mu$ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a

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QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 µL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

#### Example 13

##### Real-time Quantitative PCR Analysis of MEKK1 mRNA Levels

Quantitation of MEKK1 mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically

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between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE or FAM, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25  $\mu$ L PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM  $MgCl_2$ , 300  $\mu$ M each of dATP, dCTP and dGTP, 600  $\mu$ M of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25  $\mu$ L poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C.

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Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension). MEKK1 probes and primers were designed to hybridize to the human MEKK1 sequence, using published sequence information (GenBank accession number AF042838, incorporated herein as SEQ ID NO:1).

For MEKK1 the PCR primers were:

- 10 forward primer: GAAACTCTCAAAGGGTTGCACAA (SEQ ID NO: 2)  
reverse primer: CTGGCATACAGGTTGCCCTTCA (SEQ ID NO: 3) and  
the PCR probe was: FAM-CGTCCAGAGGAACGAATGATCAGGGA-TAMRA  
(SEQ ID NO: 4) where FAM (PE-Applied Biosystems, Foster  
City, CA) is the fluorescent reporter dye) and TAMRA (PE-  
15 Applied Biosystems, Foster City, CA) is the quencher dye.

For GAPDH the PCR primers were:

- forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 5)  
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 6) and the  
PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ  
20 ID NO: 7) where JOE (PE-Applied Biosystems, Foster City,  
CA) is the fluorescent reporter dye) and TAMRA (PE-Applied  
Biosystems, Foster City, CA) is the quencher dye.

#### Example 14

##### Northern blot analysis of MEKK1 mRNA levels

- 25 Eighteen hours after antisense treatment, cell  
monolayers were washed twice with cold PBS and lysed in 1  
mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA  
was prepared following manufacturer's recommended  
protocols. Twenty micrograms of total RNA was fractionated  
30 by electrophoresis through 1.2% agarose gels containing  
1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc.  
Solon, OH). RNA was transferred from the gel to HYBOND™-N+  
nylon membranes (Amersham Pharmacia Biotech, Piscataway,  
NJ) by overnight capillary transfer using a  
35 Northern/Southern Transfer buffer system (TEL-TEST "B")



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Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA).

- 5        Membranes were probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions with a MEKK1 specific probe prepared by PCR using the forward primer GAAACTCTCAAAGGGTTGCACAA (SEQ ID NO: 2) and the reverse  
10       primer CTGGCATACAGGTTGCCTTCA (SEQ ID NO: 3). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA). Hybridized membranes were visualized and quantitated using  
15       a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

#### Example 15

- Antisense inhibition of MEKK1 expression- phosphorothioate  
20       oligodeoxynucleotides

- In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human MEKK1 RNA, using published sequences (GenBank accession number AF042838, incorporated herein as SEQ ID  
25       NO: 1). The oligonucleotides are shown in Table 1. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. AF042838), to which the oligonucleotide binds. All compounds in Table 1 are oligodeoxynucleotides with phosphorothioate backbones  
30       (internucleoside linkages) throughout. The compounds were analyzed for effect on MEKK1 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

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Table 1

Inhibition of MEKK1 mRNA levels by phosphorothioate  
oligodeoxynucleotides

	ISIS#	REGION	TARGET	SEQUENCE	% Inhibition	SEQ ID NO.
5			SITE			
	100900	Coding	13	gctcctccgcccgcgctgc	73	8
	100901	Coding	44	ggcagccgcgcgcgcgtgc	19	9
	100902	Coding	94	cagtccgcccgcgcgcggcc	66	10
	100903	Coding	148	tcaggcagctgggtccagctc	48	11
10	100904	Coding	230	cccactccccgcgtgcgtccg	94	12
	100905	Coding	309	ccaccgactcggttaagggtgg	19	13
	100906	Coding	444	gcaaccctttgagagtttct	68	14
	100907	Coding	2400	ttcttgacagaactcaagtag	0	15
	100908	Coding	2402	cattcttgacagaactcaagt	46	16
15	100909	Coding	2404	accattcttgacagaactcaa	45	17
	100910	Coding	2406	taaccattcttgacagaactc	41	18
	100911	Coding	2408	agtaaccattcttgacagaac	0	19
	100912	Coding	2410	gtagtaaccattcttgacaga	58	20
	100913	Coding	2412	ctgtagtaaccattcttgaca	60	21
20	100914	Coding	2414	tactgtagtaaccattcttg	26	22
	100915	Coding	2416	ggtactgtagtaaccattct	47	23
	100916	Coding	2418	ggggtactgtagtaaccatt	45	24
	100917	Coding	2420	atggggtactgtagtaacca	46	25
	100918	Coding	2435	cagttttgaaaacacatggg	0	26
25	100919	Coding	2479	ctgggtgaagtgaaggaaac	24	27
	100920	Coding	2521	atttccacctcatctgcata	56	28
	100921	Coding	2568	gttgctggtgtaaagtgtct	55	29
	100922	Coding	2616	gtttccagatagttgttggg	15	30
	100923	Coding	2674	aatccttttccagttttctc	13	31
30	100924	Coding	2715	cagaaatgtcctctgaactg	50	32
	100925	Coding	2757	ttggtgaactagaaggctct	43	33
	100926	Coding	2814	gtctgccttttgtttgaacc	33	34
	100927	Coding	2879	caaggctggaaacattaatt	6	35
	100928	Coding	2957	gaatccctgaagtctatgct	48	36
35	100929	Coding	3009	gagaaaacttgcgctgtgtt	63	37
	100930	Coding	3040	ttgttttcaggacagtttct	35	38
	100931	Stop Codon	4489	tagttgatctgtataattgg	0	39
	100932	3' UTR	4512	ttgttgagcatcctgtttct	61	40
	100933	3' UTR	4550	gtagatatcaatgtggttcc	25	41
40	100934	3' UTR	4571	ctgttcagtggtcatcatggc	51	42
	100935	3' UTR	4590	ccactggcctcggtcatagc	79	43
	100936	3' UTR	4611	acatacttaggttaagggttc	44	44
	100937	3' UTR	4628	atcatgatttgtcaatcaca	38	45
	100938	3' UTR	4653	ttttgcatactgagcttagg	52	46

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100939 3' UTR 4667 gcactagtttgggcttttgc 72 47

As shown in Table 1, SEQ ID NOS 8, 10, 11, 12, 14, 16, 17, 18, 20, 21, 23, 24, 25, 28, 29, 32, 33, 34, 36, 37, 38, 40, 42, 43, 44, 45, 46 and 47 demonstrated at least 30% inhibition of MEKK1 expression in this assay and are therefore preferred.

**Example 16:****10 Antisense inhibition of MEKK1 expression- phosphorothioate 2'-MOE gapmer oligonucleotides**

In accordance with the present invention, a second series of oligonucleotides targeted to human MEKK1 were synthesized. The oligonucleotide sequences are shown in Table 2. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. AF042838), to which the oligonucleotide binds.

All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from two experiments. If present, "N.D." indicates "no data".

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Table 2

Inhibition of MEKK1 mRNA levels by chimeric  
phosphorothioate oligonucleotides having 2'-MOE wings and a  
deoxy gap

5	ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
	100940	Coding	13	gctcctccgcccgcgcctgc	93	8
	100941	Coding	44	ggcagccgcgcgcgcgtgc	25	9
	100942	Coding	94	cagtcgcgcgcgcgcggcc	61	10
10	100943	Coding	148	tcaggcagctgggtccagctc	83	11
	100944	Coding	230	cccactccccgctgcgtccg	90	12
	100945	Coding	309	ccaccgactcggtaagggtgg	71	13
	100946	Coding	444	gcaaccctttgagagtttct	87	14
	100947	Coding	2400	ttcttgacagaactcaagtag	65	15
15	100948	Coding	2402	cattcttgacagaactcaagt	58	16
	100949	Coding	2404	accattcttgacagaactcaa	72	17
	100950	Coding	2406	taaccattcttgacagaactc	62	18
	100951	Coding	2408	agtaaccattcttgacagaac	56	19
	100952	Coding	2410	gtagtaaccattcttgacaga	66	20
20	100953	Coding	2412	ctgtagtaaccattcttgaca	75	21
	100954	Coding	2414	tactgtagtaaccattcttg	72	22
	100955	Coding	2416	ggtactgtagtaaccattct	49	23
	100956	Coding	2418	ggggtagtagtaaccatt	63	24
	100957	Coding	2420	atggggtagtagtaacca	65	25
25	100958	Coding	2435	cagttttgaaaacacatggg	36	26
	100959	Coding	2479	ctggtagaagtgaaggaaac	76	27
	100960	Coding	2521	atttccacctcatctgcata	54	28
	100961	Coding	2568	gttgctcgttgtaaagtgtct	16	29
	100962	Coding	2616	gtttccagatagttgttggg	32	30
30	100963	Coding	2674	aatccttttccagttttctc	45	31
	100964	Coding	2715	cagaaatgtcctctgaactg	69	32
	100965	Coding	2757	ttgttgaaactagaaggctct	79	33
	100966	Coding	2814	gtctgccttttgtttgaacc	60	34
	100967	Coding	2879	caaggctggaaacattaatt	69	35
35	100968	Coding	2957	gaatccctgaagtctatgct	60	36
	100969	Coding	3009	gagaaaacttgcgctgtgtt	37	37
	100970	Coding	3040	ttgttttcaggacagtttct	69	38
	100971	Stop Codon	4489	tagttgatctgtataattgg	3	39
	100972	3' UTR	4512	ttgttgagcatcctgtttct	35	40
40	100973	3' UTR	4550	gtagatatcaatgtggttcc	59	41
	100974	3' UTR	4571	ctgttcagtggcatcatggc	51	42
	100975	3' UTR	4590	ccactggcctcgttcatagc	68	43
	100976	3' UTR	4611	acatacttaggtaagggttc	54	44
	100977	3' UTR	4628	atcatgatttgtcaatcaca	76	45

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100978	3' UTR	4653	ttttgcatactgagcttagg	51	46
100979	3' UTR	4667	gcactagtttgggcttttgc	69	47

As shown in Table 2, SEQ ID NOS 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 27, 28, 31, 32, 33, 34, 35, 36, 38, 41, 42, 43, 44, 45, 46 and 47 demonstrated at least 40% inhibition of MEKK1 expression in this experiment and are therefore preferred.

**Example 17****10 Western blot analysis of MEKK1 protein levels**

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to MEKK1 is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

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What is claimed is:

1. An antisense compound 8 to 30 nucleobases in length targeted to a nucleic acid molecule encoding human MEKK1, wherein said antisense compound specifically hybridizes with  
5 and inhibits the expression of human MEKK1.
2. The antisense compound of claim 1 which is an antisense oligonucleotide.
3. The antisense compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO:  
10 8, 10, 11, 12, 14, 16, 17, 18, 20, 21, 23, 24, 25, 28, 29, 32, 33, 34, 36, 37, 38, 40, 42, 43, 44, 45, 46, 47, 13, 15, 19, 22, 27, 31, 35 or 41.
4. The antisense compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO:  
15 8, 10, 11, 12, 14, 16, 17, 18, 20, 21, 23, 24, 25, 28, 32, 33, 34, 36, 38, 42, 43, 44, 45, 46 or 47.
5. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.
- 20 6. The antisense compound of claim 5 wherein the modified internucleoside linkage is a phosphorothioate linkage.
7. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified  
25 sugar moiety.
8. The antisense compound of claim 7 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.
9. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified  
30 nucleobase.
10. The antisense compound of claim 9 wherein the modified nucleobase is a 5-methylcytosine.
11. The antisense compound of claim 2 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

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12. A composition comprising the antisense compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

13. The composition of claim 12 further comprising a colloidal dispersion system.

14. The composition of claim 12 wherein the antisense  
5 compound is an antisense oligonucleotide.

15. A method of inhibiting the expression of MEKK1 in human cells or tissues comprising contacting said cells or tissues with the antisense compound of claim 1 so that expression of MEKK1 is inhibited.

10 16. A method of treating a human having a disease or condition associated with MEKK1 comprising administering to said animal a therapeutically or prophylactically effective amount of the antisense compound of claim 1 so that expression of MEKK1 is inhibited.

15 17. The method of claim 16 wherein the disease or condition is a hyperproliferative disorder.

18. The method of claim 17 wherein the hyperproliferative disorder is cancer.

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(54) Title: ANTISENSE MODULATION OF MEKK1 EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of MEKK1. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding MEKK1. Methods of using these compounds for modulation of MEKK1 expression and for treatment of diseases associated with expression of MEKK1 are provided.

WO 01/07594 A1



10/069079

Docket No.  
RTSP-0262

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ANTISENSE MODULATION OF MEKK1 EXPRESSION

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on 20 July 2000 as United States Application No. or PCT International Application Number PCT/US00/19747 and was amended on \_\_\_\_\_

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/>
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/>
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/>

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

09/359,756

23 July 1999

Patented

\_\_\_\_\_  
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\_\_\_\_\_  
(Filing Date)

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(Status)  
(patented, pending, abandoned)

\_\_\_\_\_  
(Application Serial No.)

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(Filing Date)

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(Status)  
(patented, pending, abandoned)

\_\_\_\_\_  
(Application Serial No.)

\_\_\_\_\_  
(Filing Date)

\_\_\_\_\_  
(Status)  
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Sixth inventor's signature	Date
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<160> 47

<210> 1

<211> 4693

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (1)..(4488)

<400> 1

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## 3

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Thr Leu Ser Ser Ser Ser Thr Ser Thr Ser Ser Ser Glu Asn Ser Ile	
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Lys Asp Glu Glu Glu Gln Met Cys Pro Ile Cys Leu Leu Gly Met Leu	
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Asp Glu Glu Ser Leu Thr Val Cys Glu Asp Gly Cys Arg Asn Lys Leu	
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cac cac cac tgc atg tca att tgg gca gaa gag tgt aga aga aat aga	1392
His His His Cys Met Ser Ile Trp Ala Glu Glu Cys Arg Arg Asn Arg	
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Glu Pro Leu Ile Cys Pro Leu Cys Arg Ser Lys Trp Arg Ser His Asp	
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Phe Tyr Ser His Glu Leu Ser Ser Pro Val Asp Ser Pro Ser Ser Leu	
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Arg Ala Ala Gln Gln Gln Thr Val Gln Gln Gln Pro Leu Ala Gly Ser	
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Arg Arg Asn Gln Glu Ser Asn Phe Asn Leu Thr His Tyr Gly Thr Gln	
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Gln Ile Pro Pro Ala Tyr Lys Asp Leu Ala Glu Pro Trp Ile Gln Val	
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Phe Gly Met Glu Leu Val Gly Cys Leu Phe Ser Arg Asn Trp Asn Val	
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Leu Leu Ala Asn Gly Glu Ser Thr Gly Asn Ser Gly Gly Ser Ser Gly	
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Ser Ser Pro Ser Gly Gly Ala Thr Ser Gly Ser Ser Gln Thr Ser Ile	
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tca Ser	gga Gly	gat Asp	gtg Val	gtg Val	gag Glu	gca Ala	tgc Cys	tgc Cys	agc Ser	gtt Val	ctg Leu	tca Ser	atg Met	gtc Val	tgt Cys	1872			
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gct Ala	gac Asp	cct Pro	gtc Val	tac Tyr	aaa Lys	gtg Val	tac Tyr	gtt Val	gct Ala	gct Ala	tta Leu	aaa Lys	aca Thr	ttg Leu	aga Arg	1920			
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gcc Ala	atg Met	ctg Leu	gta Val	tat Tyr	act Thr	cct Pro	tgc Cys	cac His	agt Ser	tta Leu	gcg Ala	gaa Glu	aga Arg	atc Ile	aaa Lys	1968			
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ctt Leu	cag Gln	aga Arg	ctt Leu	ctc Leu	cag Gln	cca Pro	gtt Val	gta Val	gac Asp	acc Thr	atc Ile	cta Leu	gtc Val	aaa Lys	tgt Cys	2016			
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gca Ala	gat Asp	gcc Ala	aat Asn	agc Ser	cgc Arg	aca Thr	agt Ser	cag Gln	ctg Leu	tcc Ser	ata Ile	tca Ser	aca Thr	ctg Leu	ttg Leu	2064			
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tgt Cys	att Ile	ctt Leu	gga Gly	aac Asn	caa Gln	act Thr	gaa Glu	tca Ser	aac Asn	aat Asn	tgg Trp	caa Gln	gaa Glu	ctt Leu	ctt Leu	2208			
725																730	735		
ggc Gly	cgc Arg	ctt Leu	tgt Cys	ctt Leu	ata Ile	gat Asp	aga Arg	ctg Leu	ttg Leu	ttg Leu	gaa Glu	ttt Phe	cct Pro	gct Ala	gaa Glu	2256			
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ttt Phe	tat Tyr	cct Pro	cat His	att Ile	gtc Val	agt Ser	act Thr	gat Asp	gtt Val	tca Ser	caa Gln	gct Ala	gag Glu	cct Pro	gtt Val	2304			
755																760	765		
gaa Glu	atc Ile	agg Arg	tat Tyr	aag Lys	aag Lys	ctg Leu	ctg Leu	tcc Ser	ctc Leu	tta Leu	acc Thr	ttt Phe	gct Ala	ttg Leu	cag Gln	2352			
770																775	780		
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tac Tyr	ttg Leu	agt Ser	tct Ser	gca Ala	aga Arg	atg Met	gtt Val	act Thr	aca Thr	gta Val	ccc Pro	cat His	gtg Val	ttt Phe	tca Ser	2448			
805																810	815		
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820																825	830		
agg Arg	atg Met	cgt Arg	cgc Arg	cgt Arg	ttg Leu	atg Met	gct Ala	tat Tyr	gca Ala	gat Asp	gag Glu	gtg Val	gaa Glu	att Ile	gcc Ala	2544			



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aac agc ttt tgc agg cat ctg ttc cca aca act atc tgg aaa cca cag Asn Ser Phe Cys Arg His Leu Phe Pro Thr Thr Ile Trp Lys Pro Gln 865 870 875 880			2640
aga aca gtt ccc ctt gag tgc aca gtc cat tta gag aaa act gga aaa Arg Thr Val Pro Leu Glu Cys Thr Val His Leu Glu Lys Thr Gly Lys 885 890 895			2688
gga tta tgt gct aca aaa ttg agt gcc agt tca gag gac att tct gag Gly Leu Cys Ala Thr Lys Leu Ser Ala Ser Ser Glu Asp Ile Ser Glu 900 905 910			2736
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aca aca aca aca gag caa cca aag cca atg gtt caa aca aaa ggc aga Thr Thr Thr Thr Glu Gln Pro Lys Pro Met Val Gln Thr Lys Gly Arg 930 935 940			2832
ccc cac agt cag tgt ttg aac tcc tct cct tta tct cat cat tcc caa Pro His Ser Gln Cys Leu Asn Ser Ser Pro Leu Ser His His Ser Gln 945 950 955 960			2880
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cca gct ggc act gca aca gat gtc tct aag cat aga ctt cag gga ttc Pro Ala Gly Thr Ala Thr Asp Val Ser Lys His Arg Leu Gln Gly Phe 980 985 990			2976
att ccc tgc aga ata cct tct gca tct cct caa aca cag cgc aag ttt Ile Pro Cys Arg Ile Pro Ser Ala Ser Pro Gln Thr Gln Arg Lys Phe 995 1000 1005			3024
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aaa tgt gat gac agc ttt ggc ttg agc agc aat agt agt aat tgc tgt			3264

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Lys Cys Asp Asp Ser Phe Gly Leu Ser Ser Asn Ser Ser Asn Cys Cys	
1075 1080 1085	
tat acc agt gac gag aca gtg ttc acc cca gta gag gag aaa tgc aga	3312
Tyr Thr Ser Asp Glu Thr Val Phe Thr Pro Val Glu Glu Lys Cys Arg	
1090 1095 1100	
tta gat gtc aat aca gag ctc aac tcc agt att gag gac ctt ctt gaa	3360
Leu Asp Val Asn Thr Glu Leu Asn Ser Ser Ile Glu Asp Leu Leu Glu	
1105 1110 1115 1120	
gca tct atg cct tca agt gat aca aca gta act ttt aag tca gaa gtt	3408
Ala Ser Met Pro Ser Ser Asp Thr Thr Val Thr Phe Lys Ser Glu Val	
1125 1130 1135	
gct gtc ctg tct cct gaa aag gct gaa aat gat gat acc tac aaa gat	3456
Ala Val Leu Ser Pro Glu Lys Ala Glu Asn Asp Asp Thr Tyr Lys Asp	
1140 1145 1150	
gat gtg aat cat aat caa aag tgc aaa gag aag atg gaa gct gaa gaa	3504
Asp Val Asn His Asn Gln Lys Cys Lys Glu Lys Met Glu Ala Glu Glu	
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gaa gaa gct tta gca att gcc atg gca atg tca gcg tct cag gta gcc	3552
Glu Glu Ala Leu Ala Ile Ala Met Ala Met Ser Ala Ser Gln Val Ala	
1170 1175 1180	
ctc ccc ata gtt cct cag ctg cag gtt gaa aat gga gaa gat atc atc	3600
Leu Pro Ile Val Pro Gln Leu Gln Val Glu Asn Gly Glu Asp Ile Ile	
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att att caa cag gat aca cca gag act cta cca gga cat acc aaa gca	3648
Ile Ile Gln Gln Asp Thr Pro Glu Thr Leu Pro Gly His Thr Lys Ala	
1205 1210 1215	
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Lys Gln Pro Tyr Arg Glu Asp Thr Glu Trp Leu Lys Gly Gln Gln Ile	
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Gly Leu Gly Ala Phe Ser Ser Cys Tyr Gln Ala Gln Asp Val Gly Thr	
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Gly Thr Leu Met Ala Val Lys Gln Val Thr Tyr Val Arg Asn Thr Ser	
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Ser Glu Gln Glu Glu Val Val Glu Ala Leu Arg Glu Glu Ile Arg Met	
1265 1270 1275 1280	
atg agc cat ctg aat cat cca aac atc att agg atg ttg gga gcc acg	3888
Met Ser His Leu Asn His Pro Asn Ile Ile Arg Met Leu Gly Ala Thr	
1285 1290 1295	
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Cys Glu Lys Ser Asn Tyr Asn Leu Phe Ile Glu Trp Met Ala Gly Gly	
1300 1305 1310	

7

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Ser Val Ala His Leu Leu Ser Lys Tyr Gly Ala Phe Lys Glu Ser Val
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gtt att aac tac act gaa cag tta ctc cgt gga ctt tcg tat ctc cat      4032
Val Ile Asn Tyr Thr Glu Gln Leu Leu Arg Gly Leu Ser Tyr Leu His
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gaa aac caa atc att cac aga gat gtc aaa ggt gcc aat ttg cta att      4080
Glu Asn Gln Ile Ile His Arg Asp Val Lys Gly Ala Asn Leu Leu Ile
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gac agc act ggt cag aga cta aga att gca gat ttt gga gct gca gcc      4128
Asp Ser Thr Gly Gln Arg Leu Arg Ile Ala Asp Phe Gly Ala Ala Ala
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agg ttg gca tca aaa gga act ggt gca gga gag ttt cag gga caa tta      4176
Arg Leu Ala Ser Lys Gly Thr Gly Ala Gly Glu Phe Gln Gly Gln Leu
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ctg ggg aca att gca ttt atg gca cct gag gta cta aga ggt caa cag      4224
Leu Gly Thr Ile Ala Phe Met Ala Pro Glu Val Leu Arg Gly Gln Gln
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Tyr Gly Arg Ser Cys Asp Val Trp Ser Val Gly Cys Ala Ile Ile Glu
      1410                      1415                      1420

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Met Ala Cys Ala Lys Pro Pro Trp Asn Ala Glu Lys His Ser Asn His
      1425                      1430                      1435                      1440

ctt gct ttg ata ttt aag att gct agt gca act act gct cca tcg atc      4368
Leu Ala Leu Ile Phe Lys Ile Ala Ser Ala Thr Thr Ala Pro Ser Ile
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cct tca cat ttg tct cct ggt tta cga gat gtg gct ctt cgt tgt tta      4416
Pro Ser His Leu Ser Pro Gly Leu Arg Asp Val Ala Leu Arg Cys Leu
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gaa ctt caa cct cag gac aga cct cca tca aga gag cta ctg aag cat      4464
Glu Leu Gln Pro Gln Asp Arg Pro Pro Ser Arg Glu Leu Leu Lys His
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cca gtc ttt cgt act aca tgg tag ccaattatac agatcaacta cgtagaaaca      4518
Pro Val Phe Arg Thr Trp
      1490                      1495

ggatgctcaa caagagaaaa aaaacttgtg gggaaccaca ttgatatac cggccatgat      4578

gccactgaac agctatgaac gaggccagtg gggaaccctt acctaagtat gtgattgaca      4638

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